STAIN TECHNOLOGY

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PROGRESS IN THE STANDARDIZATION OF STAINS

BIOLOGICAL STAINS IN TIME OF WAR

The Biological Stain Commission was conceived as the result of the World War. Now it faces another European war, and it is interesting to notice the differences in the situation.

In 1914 there were no American-made dyes. All stains used in American laboratories were of German manufacture, mostly the product of two concerns, which had originated from the efforts of a single man and were often confused in this country so that the two lines of German stains were thought to be one. When the United States entered the war the various new laboratories which were set up at that time were unable to obtain German dyes and found the American products unreliable. It was to meet this situation and to put the use of stains on a more scientific basis that the Stain Commission was established.

Today, the situation could hardly be more different. Nearly every large nation has its dye industry, and that of the United States is unsurpassed. Thanks to the cooperation of the manufacturers on the one hand and numerous scientists on the other, the production of biological stains is now on a scientific basis; the manufacturers know what biologists want and the biologists know how to make any new requirements they may have intelligible to the industry. Furthermore, practically all stains and every intermediate needed for their manufacture can be obtained from American sources. There probably are *no* exceptions to this statement, altho there are a few of the less common dyes and compound stains which have been largely imported lately because of difficulties that have been obtained with some of the American products. Among these have been brilliant cresyl blue and Giemsa stain. It is interesting, by the way, to note that American stain companies report a greatly increased demand for these two particular stains since September first.

There has, as a matter of fact, been a general stimulation to the stain industry since summer. This has been reflected by the orders the stain companies have sent to the Stain Commission for certification labels. The increased demand began in August, so it could not

have been due wholly to the shutting off of the European sources of supply. September, and then again October, broke all preceding records in the demand for certification labels. This has been very gratifying, especially now that the Commission is no longer being fianaced by the chemical foundation. If the increased demand proves to be more than temporary, the Commission may become self-supporting. Users of stains can help make it permanent by taking pains to see that their institutions order Commission certified stains.

H. J. Conn

STAINS RECENTLY CERTIFIED

In the table below is given a list of the batches of stains approved since the last one listed in the October number of this Journal.

Stains Certified Sept. 1, 1939 to Nov. 30, 1939*

Name of dye	Certification No. of batch	Dye Content	Objects of tests made by Commission†	Date approved
Methylene blue chloride	NA-14	87%	As histological and bacteriological stain, and as constituent of blood stains	Sept. 8, 1939
Brilliant cresyl blue	NV-17	57%	As vital stain for blood	Sept. 30, 1939
Sudan IV	LZ-2	81%	As stain for fat	Oct. 13, 1939
Indigo carmine	LI-1	83%	As histological stain	Oct. 13, 1939
Fast green FCF	CGf-1	90%	As histological and cytological counterstain	Oct. 13, 1939
Methylene blue chloride	DA-4	84%	As histological and bacteriological stain, and as constituent of blood stains	Nov. 1, 1939
Basic fuchsin	NF-32	93%	For general staining, the Feulgen reaction and in bacteriological media	Nov. 2, 1939
Brilliant cresyl blue	NV-18	63%	As vital stain for blood	Nov. 8, 1939
Sudan IV	NZ-11	64%	As stain for fat	Nov. 10, 1939
Janus green B	NJ-8	73%	As vital stain for blood	Nov. 15, 1939
Crystal violet	MC-1	93%	As histological, cytological, bacteriological stain, and in bacteriological media	Nov. 21, 1939
Eosine B.	NEb-8	80%	As histological counterstain	Nov. 24, 1939
Methylene azure	LAz-1	63%	As histological stain and as constituent of blood stains	Nov. 27, 1939

Special note:—Attention is called to the two samples of brilliant cresyl blue certified within about six weeks of each other. Both were submitted by the same company. Such a short interval between samples submitted has never occurred before; it seems to indicate a suddenly increased demand for Commission Certified samples of this particular dye.

*The name of the company submitting any one of these dyes will be furnished on request.

†It is not to be inferred that these are the only uses for which each of these samples may be employed. The Commission ordinarily tests each dye for such of its common uses as seem to give the most severe check as to its staining value. Certification does not in any instance, however, imply approval for medicinal use.

A STANDARDIZED TECHNIC FOR SAFRANIN O[1]

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ABSTRACT.—**A method for control of staining with Safranin O is described. The procedure is as follows:**

Overstain the sections, freed of paraffin, 4 hours or more in 0.1% solutions of either light green S F or fast green F C F in 50% alcohol. These solutions are adjusted to pH 2.4 with 0.1 *N* HCl. Rinse in distilled water.

Destain at least 30 minutes in Sörensen's Buffer pH 8. Rinse in distilled water.

Overstain in 0.1% Safranin O, 4 hours or more. Rinse in distilled water.

Destain 15 minutes in 0.01 *N* HCl (pH 2) or in 0.001 *N* HCl (pH 3) depending on whether light green or fast green, respectively, is the counterstain. The acid solutions are freshly prepared from a stock solution of 0.1 *N* acid. Rinse in distilled water.

Dehydrate in two changes of dioxan, pass thru xylol and mount in balsam.

Safranin O, one of the most valuable nuclear stains, has always presented difficulties to the histologist; it has given extremely variable results (Conn, 1936, p. 86). At least a part of this inconstancy has been due to the solubility of the stain in alcohol. Destaining and dehydrating are usually performed simultaneously and often too much of the stain washes out before the preparation is mounted. The aim of the present work has been to obviate this phase of the difficulty by controlling the destaining process in an aqueous solution of definite pH and by dehydrating in dioxan (Guyer, 1936, p. 64). The author is grateful to Dr. Petrunkevitch, who devised the method (Petrunkevitch, 1937), and under whose supervision the work was done.

It was first necessary to find the optimum pH to use for destaining. A curve (Fig. 1) was prepared to enable one to make up quickly a series of 0.1% aqueous solutions of the stain from pH 2 to pH 10. The procedure was to add varying amounts of 0.1 *N* HCl or NaOH to 25 ml. samples of a stock solution of 0.2% Grübler's Safranin O and to dilute with distilled water to 50 ml. in a volumetric flask. A Beckman glass-electrode pH-meter was used in pH measurements,

[1]Contribution from the Osborn Zoological Laboratory, Yale University.

and all the readings were taken at 23° C. From the curve a stain series was set up ranging from pH 2 to pH 9.

Since staining affinity varies widely with the fixation employed, four representative fixing fluids were used: Petrunkevitch's paranitrophenol–cupric-nitrate–nitric (Guyer, p. 34), Zenker's bichromate–sublimate–sodium-sulphate–acetic (Lee, 1937, p. 46), Bouin's picro-

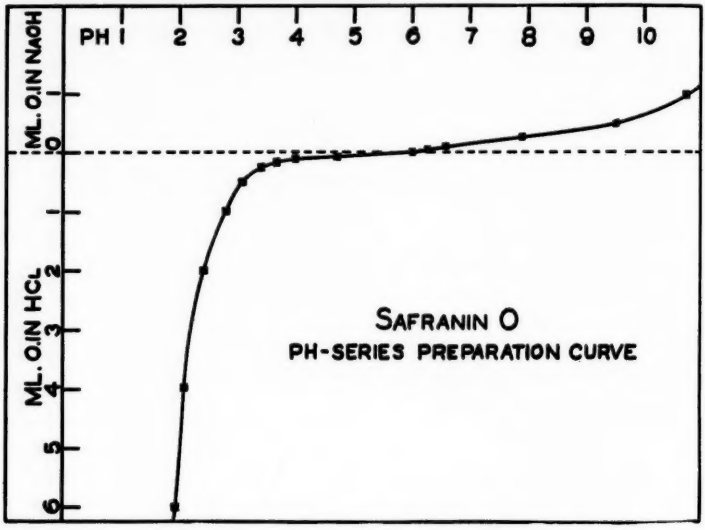

Fig. 1. Curve showing amount of 0.1 *N* acid or alkali needed to make up 50 ml. 0.1% Safranin O at various pH-values.

formol-acetic (Lee, p. 58), and Petrunkevitch's sublimate-nitric-acetic-alcohol (Lee, p. 45). The tissue, intestine of the leopard frog, was fixed in these fluids and sectioned at 10μ in paraffin. The sections, freed of paraffin, were stained in the pH-series overnight to insure completion of the staining reaction. Each was then rinsed in an HCl or NaOH solution of approximately the same H-ion concentration as the stain, dehydrated in two changes of dioxan, passed thru xylol, and mounted in balsam. Treatment preliminary to staining may be with either alcohol or dioxan, but the latter is quicker.

From the results (Figs. 2–5) it can be seen that the greatest differentiation between nuclear and cytoplasmic staining is in each case at pH 3 or 4 and that nearly maximal staining occurs at pH 6, the stain prepared with no addition of acid or alkali. At pH 2 the material fixed in Bouin's or Zenker's fluids shows no nucleo-cytoplasmic differentiation; maximal differentiation is reached at pH 4. The Bouin-

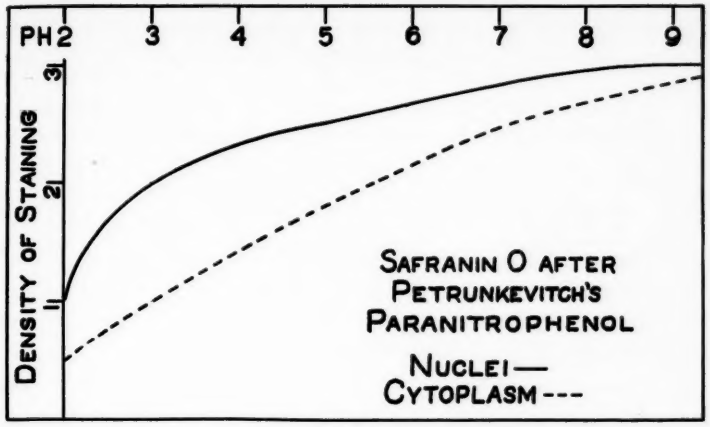

Figure 2.

fixed sections were unusual in that the secretion-filled goblet cells of the lining epithelium had a stronger affinity for the stain than did the nuclei—taking the stain even at a reaction of pH 2. The tissue fixed in Petrunkevitch's fluids showed its highest differentiation at pH 3. No color comparator was used in judging differentiation, but the results were sufficiently clear-cut so that the estimations of relative density designated on the curves as 1, 2, and 3 for slight, medium and maximum, are fairly accurate.

On the basis of these results the destaining method was attempted, keeping in mind the rule that the capacity of retaining a stain in a de-

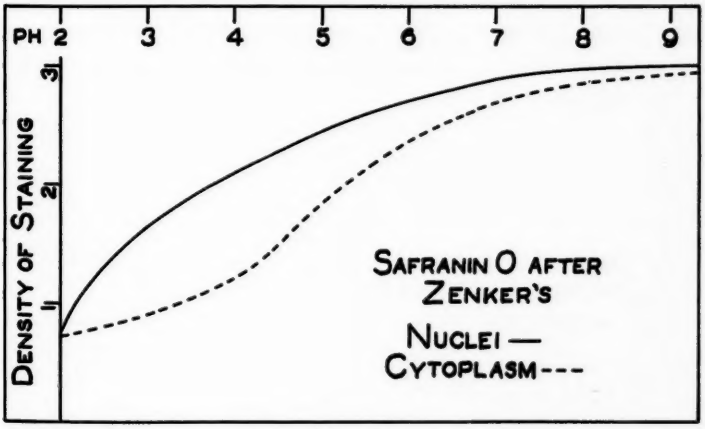

Figure 3.

staining fluid of a given pH-value is not the same as the staining affinity at that pH-value. Inasmuch as the stain without treatment by acid or alkali gave nearly maximal affinity, it was used untreated for the preliminary overstaining. Destaining at pH 2 (0.01 *N* HCl, prepared by diluting 5 ml. 0.1 *N* HCl to 50 ml. in a volumetric flask) gave the following results: Zenker-fixed material, no differentiation; Bouin-fixed, goblet cells only; Petrunkevitch's sublimate or *p*-nitrophenol-fixed, excellent differentiation, only the chromatin retaining

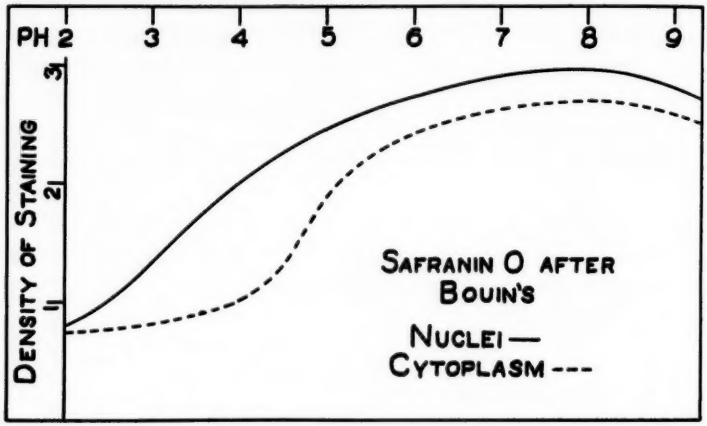

Figure 4.

the stain. Optimum destaining for Bouin- or Zenker-fixed tissue was achieved at pH 3, but the Bouin-fixed material never gave excellent differentiation. Of the Petrunkevitch fixatives the *p*-nitrophenol gave slightly the more striking contrast. Tho the destaining process is theoretically independent of the time element, provided completion of the destaining reaction is reached, prolonged exposure to pH 2 tends to make the sections slip off the slide; 15 minutes is sufficient for optimal destaining in acid.

Two green counterstains were successfully employed, light green S F (Coleman and Bell Co.) and fast green F C F (National Aniline and Chemical Co.). These gave optimum results when the sections were overstained in 0.1% solution in 50% alcohol at pH 2.4 and destained at pH 8 with Sörensen's phosphate buffer. Each of the acid staining solutions was made up by taking 10 ml. of 0.5% aqueous solution of the stain, adding 25 ml. of absolute alcohol and 4 ml. of 0.1 *N* HCl, and diluting with water to 50 ml. Best results were obtained by applying the counterstain before staining with Safranin O.

Of the two greens, light and fast, the latter is much the better from the point of view of permanency. Safranin O, however, has a weaker affinity for sections previously counterstained by fast green; so if the latter is used the final destaining is better effected at pH 3 (0.001 *N* HCl).

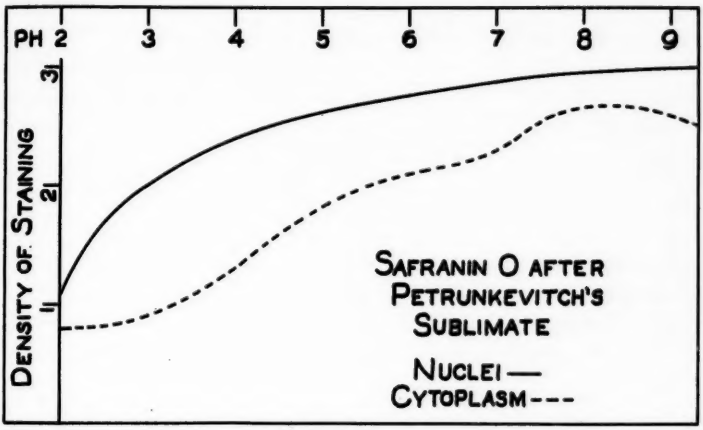

Figure 5.

Not all stains are amenable to the treatment described here. It may appropriately be reported that crystal violet gives negative results on application of this technic. One can tell only by experiment whether or not a particular stain will react favorably to the treatment. As has been stated, the procedure for Safranin O was developed using a sample of Grübler's ("water soluble"). The method has since been satisfactorily applied to a second batch of Grübler's and to a sample from the National Aniline and Chemical Co. (Certification No. NS-9, dye content 97%), so it should be generally applicable to Safranin O.

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STAINING PARAFFIN SECTIONS WITH PROTARGOL

5. CHLORAL HYDRATE MIXTURES, WITH AND WITHOUT FORMAMIDE, FOR FIXING PERIPHERAL NERVES.[1]

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ABSTRACT.—**A series of experiments was directed toward finding a means of improving fixation of mammalian glandular tissue and peripheral nerves with chloral hydrate. Specimens from cat, dog, rat, guinea pig, and man were fixed in solutions of 5–15% chloral hydrate in ethyl, methyl, and propyl alcohols, both pure and diluted with varying amounts of water. Modifiers were added, including acids, alkalies, alkaloids, amines, formamide, pyridine, piperidine, and formalin. The sectioned material was stained by the 2-hour method ($AgNO_3$-protargol) described previously (Davenport et al., 1939). The acidification of alcoholic chloral hydrate mixtures was deleterious to fixation but alkalinization was not. Among the modifiers, formamide was the one which showed definite improvement of fixation. A 10% solution of formamide alone in 50% ethyl alcohol gave good fixation and staining of peripheral nerve trunks, but addition of 5–7% chloral hydrate to this mixture improved staining. Treatment with 1% ammoniated alcohol after fixation and before embedding was of no value in section staining. Block stains were not tried.**

In the preceding paper of this series (Davenport, McArthur, and Bruesch, 1939) a two-hour method for staining nervous tissue was described. The method was worked out chiefly on spinal nerve roots, tissue from the central nervous system, and sympathetic trunks. The use of acid mixtures recommended for fixing central nervous tissue and roots has not proved very satisfactory for peripheral nerve trunks and is particularly bad for glandular tissue. The present study has been directed toward improvement in fixation and differentiation of the more peripherally located nerves by the use of neutral or alkaline fixatives based on mixtures of chloral hydrate, alcohol and water. Chloral hydrate was chosen because it has been

[1]Contribution No. 303 from the Department of Anatomy, Northwestern University Medical School.

[2]Submitted by E. W. Bank as a partial requirement for the degree, Master of Science, to the Graduate School of Northwestern University.

TABLE 1. LIST OF FIXATIVES*

No.	Chloral hydrate	Modifier	Alcohol		Water
1	15	0	methyl celosolve,	100	0
2	15	0	Id.	70	30
3	15	0	Id.	30	70
4	15	0	*n*-propyl,	70	30
5	15	0	abs. ethyl,	100	0
6	15	0	" "	70	30
7	15	0	" "	30	70
8	15	0	methyl,	100	0
9	15	0	"	70	30
10	15	0	"	30	70
11	12	amm. w., 0.5‡	*n*-propyl,	60	40
12	12	quinine hydrochloride, 2	"	60	40
13	12	glacial acetic, 5	"	60	40
14	12	monochloracetic, 0.2	"	60	40
15	12	" 0.2	ethyl	60	40
16	12	0	pyridine,	50	50
17	12	amm. w., 0.25	propyl,	50	
			ethyl	5	45
18	12	piperidine, 0.25	propyl,	50	
			ethyl	5	45
19	12	*n*-butylamine, 0.25	propyl,	50	
			ethyl	7.5	42.5
20	12	formamide, 5	propyl,	50	
			ethyl,	5	45
21	12	ethanolamine, 3.5	propyl,	50	
			ethyl	5	45
22	12	0	propyl,	50	
			ethyl,	15	35
23	7	formamide, 15	propyl,	30	50
24	7	amm. w., 0.25			
		formamide, 15	propyl,	30	50
25	0	formamide, 20	butyl,	15	
			ethyl	5	60
26	0	formamide, 20	butyl,	15	
			py,	5	60
27	0	formamide, 20		0	80
28	0	formamide, 20			
		formalin, 2		0	78
29	5	brucine, 5	*n*-propyl,	50	50
30	0	" 5	"	50	50
31	5	" 5	methyl,	100	0
32**	5	Py, 20	ethyl,	40	40
33***	5	amm. w., 0.2	ethyl,	50	50
34†	3	formalin, 12	ethyl,	50 pH	50
35	7	formamide, 7	propyl,	35–4.9	50
36	7	" 7+amm. w., 1.0	"	35–8.9	50
37	7	" 7 " " 0.1	"	35–7.4	50
38	7	" 7+trichlor-acetic acid, 0.01	"	35–4.7	50

*Formamide, piperidine, *n*-butylamine, ethanolamine, and *n*-propyl alcohol were obtained from Eastman Kodak Co., Rochester, N. Y. Protargol was obtained from Winthrop Chemical Co., New York, N. Y.

**d'Ancona's fluid No. 1

***d'Ancona's fluid No. 2

†One of Cajal's formulae.

‡Abbreviations: amm. w., strong ammonia water; Py, pure pyridine (Merck).

TABLE 1. LIST OF FIXATIVES (continued)

No.	Chloral hydrate	Modifier	Alcohol		Water
39	7	formamide, 7+trichlor-acetic acid, 0.1	"	35–4.1	50
40	0	formamide, 7	"	35–4.9	50
41	7	" 7	ethyl	35–4.9	50
42	0	" 7	"	35–5.0	50
43	7	" 0	"	50	50
44	0	formamide, 10	"	50	50
45	7	" 10	"	50	50
46	7	" 10+Py, 15§	"	35	50
47	7	" 10+Py, 15+ formalin, 4	"	35	50
48	7	formamide, 10		0	100
49	7	" 10	methyl,	50	50
50	7	" 10	propyl,	50	50
51	0	0	ethyl,	50	50
52	0	0	propyl,	50	50

§Py = pure pyridine (Merck).

used successfully by previous investigators (Cajal 1907, 1910, 1929; d'Ancona 1925; and Perez 1932) for nerve endings when block staining of the Cajal type was used.

Material and Methods. Nearly all the experiments were made by using 6–10 fixatives in a group and fixing in each of these a piece of nerve and two pieces of glandular tissue, such as pancreas, thyroid, adrenal and salivary glands, from the same animal. Represented in the series were 7 cats, 4 dogs, 6 rats, 1 guinea pig, and 1 human. The tissues were fixed by immersion, and the time was about 20 hours. Embedding in paraffin, cutting, and mounting the sections on the slide were done in the routine manner. Paraffin was removed and the slides passed to water thru graded alcohol, impregnated 1 hour at 60° C. with 5% aqueous $AgNO_3$ solution, washed in 3 changes of distilled water 0.5 minutes each and put into 0.2% protargol solution at 27° C. for 1 hour. Following impregnation a rinse of 1 or 2 seconds in distilled water was given and the protargol reduced by amidol-sulfite solution (amidol, 0.1 g.; $NaHSO_3$, 1.0 g.; Na_2SO_3, 10 g.; and water, 100 cc.) for 1–2 minutes. The reducing solution was made up immediately before using by adding the dry amidol to a stock solution of the sulfites. The slides were washed carefully to remove all reducing solution and toned in a 0.1% solution of $AuCl_3$ for about 5 minutes, washed again and, if the stain were light, reduced 0.5 minutes with a 0.5% amidol solution; if dark, reduced 5 minutes with a mixture of oxalic acid and formalin (oxalic acid, 2 g.; formalin, 1 cc.; water 100 cc.). To insure the removal of unreduced AgCl, a

5–10% "hypo" solution should be used after the second reduction, but this was not done in a routine way, because the preparations were kept only a short time. A final washing followed by dehydration and covering in balsam completed the staining. The procedure has a few minor modifications of the original method.

Fixatives: The 52 fixatives listed in Table 1 are about half of the total number tried, but include those which are characteristic representatives of the various modifiers used. We had in mind the following questions:

1. Is there any advantage of methyl or *n*-propyl alcohol over ethyl?
2. What is the proper water content of these alcohols when used with chloral hydrate?
3. What is the effect of adding acid or base?
4. Does the addition of amines, amine-like substances, or alkaloids improve fixation or subsequent staining with silver?

In addition to the fixatives in Table 1, 12% chloral hydrate solutions in isopropyl, allyl, *n*-butyl, isobutyl, and octyl alcohols and in dioxan were tried. With the exception of isopropyl alcohol, excessive shrinkage occurred with their use. Our findings with regard to the higher alcohols agree with Cajal's (1907) observations on their lack of utility in similar fixing solutions for staining in the block.

In one series of tissue samples (fixatives 43–51) the specimens were cut in two after fixation and one half given a further treatment of 24 hours in 1% ammoniated alcohol. Such treatment seemed to be of no value or even deleterious to both staining and fixation, as shown by paler stains and greater shrinkage.

Methyl cellosolve and dioxan were poor substitutes for alcohol.

Results and Discussion: No advantage of methyl or propyl alcohol over ethanol appeared consistently. When used without dilution with water, propyl alcohol caused more generalized shrinkage than either methanol or ethanol. It was found that 10–15% solutions of chloral hydrate in mixtures of water and propyl alcohol separated into two layers when the water content exceeded 40%, but these could be made into perfect solutions by the addition of a small amount of ethyl alcohol, ethanolamine, formamide, ammonia or other modifiers used in fixatives 17 to 22. Ethyl and methyl alcohols gave perfect solutions when mixed with any amount of water.

The answer to question 2, regarding water content of the fixative, appears to be that only methyl alcohol can be used pure with impunity as a solvent for chloral hydrate in fixation. Ethyl and propyl alcohols cause excessive shrinkage when so used. Ethanol as 50 and 70% mixtures with water, and propanol diluted to about 40% with

sufficient formamide (fixative 35) to homogenize the solution were definitely more satisfactory than higher concentrations.

The addition of acids was deleterious, but the addition of ammonia, piperidine, pyridine, and butylamine in small amounts was not. Addition of acids, except in relatively minute amounts (fixative 38), caused serious cracking of the tissue, especially peripheral nerves, with the result that cross sections presented a mosaic-like pattern caused by longitudinal fissures—a type of artifact commonly seen after fixation in neutral formalin. In some specimens the addition of alkali favored the subsequent differentiation of nerve fibers in glands, but in others this effect was not seen. It appeared to have no effect on fixation *per se*.

Of all the modifiers tried, only one showed a consistently beneficial effect both on fixation and on subsequent staining. This was formamide, and while it was used by Cajal (1907) as a 4% solution in 96% alcohol, we have found no mention of its use in conjunction with chloral hydrate. This substance, altho it has a chemical formula like an amine, has properties more nearly resembling urea in that it is able to denature protein and is not alkaline. As an addition to chloral hydrate solutions, it seems to be well worth while. Used alone (without chloral hydrate) in water-ethanol or water-propanol mixtures (fixatives 40 and 44) it has given superior fixation and strong staining with silver. The addition of chloral hydrate to the mixture, however, appears to give some improvement in the differentiation of nerve fibers.

The use of formalin (fixatives 28, 34, and 47) was deleterious both to fixation and staining.

We wish to suggest that fixatives having formulas the same as, or similar to, those given in fixatives 23, 24, 35, 36, 41, 45, 49 and 50 are worthy of trial in the staining of peripheral nerve trunks, particularly when enumeration of fibers is desired.

Results with glandular material have been only partially successful; hence the use of chloral hydrate mixtures, altho a great improvement over acid fixatives, leaves much to be desired in section staining with the silver-nitrate-protargol method.

We wish to acknowledge aid from the National Youth Administration to E. W. Bank.

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A PROCEDURE FOR STAINING FILAMENTOUS ALGAE AND FUNGI ON THE SLIDE

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The Venetian Turpentine method for mounting delicate structures such as filamentous algae requires so much handling of the bulk material that tangling, twisting, and excessive breaking of the filaments is almost unavoidable. The conjugating filaments of forms which show scalariform conjugation are likely to be separated and otherwise distorted. Due to matting of the filaments, uniform destaining is difficult of accomplishment, and satisfactory teasing-out of the matted filaments in the thick Venetian Turpentine just prior to covering is difficult or impossible.

The following procedure, based upon the use of a modification of Haupt's[1] gelatine fixative, has been used successfully by the writer in overcoming these difficulties and in shortening the preparation time.

Dissolve 1.5 g. of a good grade of gelatin in 100 ml. of distilled water at 30–35° C. Add 5 ml. of glycerin and 2 g. of phenol. Smear a thin film of the fixative upon a warm slide.

After killing and washing the bulk material in running water remove enough for a slide with fine forceps. Place this upon the smeared slide with a "dragging" movement, beginning at one end of the slide and pulling the filaments toward the other end. The water which adheres to the filaments is sufficient for floating them into position; a large quantity of water such as is used for floating paraffin ribbons is unnecessary and undesirable. Most of the filaments will thus arrange themselves in a free and parallel fashion. Conjugating filaments can be mounted in this manner without damage. If desired, long ends extending beyond the area to be covered may be turned in beside the main mass of filaments. Invert the slide over a shallow dish containing a small quantity of commercial (40%) formaldehyde and cover with an inverted dish or low bell jar. Glass staining dishes of the low, rectangular form, slightly less than 3 inches wide, serve this purpose well. Leave the slides thus exposed to the fumes of formaldehyde for ½ hour. Wash slides in distilled water, 5–10 minutes. They are now ready for staining in aqueous stain or for the alcohol series if an alcoholic stain is to be used. Clear in xylene and mount in balsam.

[1]Haupt, A. W. 1930. A gelatine fixative for paraffin sections. Stain Techn. 5, 97–8.

The following schedule has given good preparations of *Spirogyra:*

1. Kill and fix in chromo-acetic acid (1% chromic acid, 3% glacial acetic acid), 12 hours.

2. Wash in running water, 12 hours.

3. Fix material to slide as described above. Wash in distilled water, 5–10 minutes. (Slides may at this point be run up to 85% alcohol for hardening and returned to water.)

4. Mordant in 2% aqueous solution of ferric ammonium sulfate, 4 hours. Wash in several changes of water, ½ hour.

5. Stain in 0.5% aqueous hematoxylin, 12 hours. Wash in water (2 or 3 changes), ½ hour.

6. Destain with 1% aqueous solution of ferric ammonium sulfate, about 1 hour. Wash in water, ½ hour.

7. Dehydrate by closely graded series of alcohols to 95%.

8. Counterstain with 0.5% orange G in 95% alcohol, 15–30 seconds.

9. Complete dehydration in absolute alcohol, 1–2 minutes. Clear in xylene. Mount in balsam.

Other filamentous algae with this and other combinations of stains have given good preparations. Miss Alma Whiffen of this laboratory has used successfully the present technic for mounting and staining cultures of *Achlya* grown on hemp seed.

FURTHER EXPERIMENTS WITH THE MASSON TRICHROME MODIFICATION OF MALLORY'S CONNECTIVE TISSUE STAIN[1]

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ABSTRACT.—**Several dyes, notably ponceau 2R, azofuchsin 3B, nitrazine yellow, and Biebrich scarlet may replace imported "ponceau de xylidine" in the Masson ponceau acid fuchsin mixture. Of these Biebrich scarlet appears to be the best and may be used without acid fuchsin.**

A mixture of equal parts of 5% solutions of phosphomolybdic and phosphotungstic acids is much superior to either acid alone and gives adequate mordanting in 1 minute at 22° C.

With the fast green modification, times in plasma and fiber stains can be reduced to 2 minutes each. With anilin blue a 4-minute plasma stain is required. One-minute final differentiation in 1% acetic acid is adequate.

Primary mordanting of formalin material may be accomplished by 5 minutes in saturated aqueous mercuric chloride or 2 minutes in saturated alcoholic picric acid. Three minutes washing in running water is required after these mordants.

While our previous studies (Lillie, 1938) and those of Goldner (1938) indicated that the trichrome method could be adapted to formalin material and considerably shortened, the length of time (nearly an hour) and the necessity for incubation required by the best of our modifications were still a bar to the frequent or routine use of the method. Moreover, the necessity for an imported "ponceau de xylidene" for the proper execution of the method gave rise to some difficulties in prompt procurement of the dye. Consequently, we determined to test a number of other dyes as to their availability for plasma stains in place of "ponceau de xylidene," and to endeavor to further shorten the procedure.

The following basic technic was used in experiment 9.[2] Bring paraffin sections of formalin fixed tissues to water as usual. (1) Treat in mordant No. 1 (saturated aqueous picric acid) 10 minutes at 58° C. (2) Wash 15 minutes in running water. (3) Apply Wei-

[1]Contribution from Division of Pathology, National Institute of Health.

[2]Experiments 1 to 8 are included in the previous report (1).

gert's acid iron chloride hematoxylin, 6 minutes. (4) Rinse in water. (5) Stain 5 minutes in plasma stain. (6) Rinse in distilled water. (7) Apply mordant No. 2 (1% phosphomolybdic acid) 10 minutes at 58° C. (8) Stain in 2.5% fast green FCF in 2.5% acetic acid, 5 minutes. (9) Differentiate in 1% acetic acid for 2 minutes. (10) Carry thru alcohol, acetone, acetone+xylene, and xylene to salicylic balsam. The plasma stains were made by mixing 9 cc. of 1% solution of the various dyes with 1 cc. of 1% acid fuchsin and 0.1 cc. glacial acetic acid.

The best plasma stains were given by ponceau 2R, nitrazine yellow, Biebrich scarlet and azofuchsin 3B. Almost as good were Bordeaux red, chromotrope 2R, azofuchsin G, orange G and crocein. Still quite good were "ponceau de xylidene," azofuchsin (C.I. 30), azofuchsin 4G, eosin Y and chrysoidin.[3] Distinctly inferior were azofuchsin B, azofuchsin 6B (C.I. 57), azofuchsin 6B or GN (C.I. 154), methyl eosin and erythrosin. Phloxine gave the poorest effect of any. (The sources of these dyes are given in Table 1.) Appendix, pneumonic lung and a nephrosclerotic kidney were used as test objects.

Having noted the statement in Thorpe and Linstead (1933) "that the lakes of complex acids containing both tungsten and molybdenum were much faster than those containing either metal alone" (English patent 216486, 1923), it was decided to compare a mixture of phosphotungstic and phosphomolybdic acids as second mordant with the two acids used alone. In experiment 10 the usual technic was followed, employing Biebrich scarlet with acid fuchsin as the plasma stain. Second mordants were as follows: 1% phosphomolybdic acid, 10 minutes at 58° C.; 1% phosphotungstic acid, 10 minutes at 58° C.; and a mixture of equal parts of 1% solutions of phosphomolybdic and phosphotungstic acids for 5 and 10 minutes at 58° C. and for 5, 10, 15, 30 and 60 minutes at room temperature.

Phosphotungstic acid alone gave distinctly inferior results, but the mixture was excellent at 58° C. for either 5 or 10 minutes and at room temperature when the time was 10 or more minutes.

As Goldner had found he could shorten the phosphomolybdic mordanting time by using more concentrated solutions at room temperature, we decided to try in experiment 11 the effect of raising the concentration of the mixed acids to 5%. The Biebrich scarlet variant was used as before. A control of 1% phosphomolybdic and phosphotungstic acids at room temperature for 10 minutes was used,

[3]This dye was labeled chrysoidin Y, but was shown not to agree with the characteristics of that dye.

and trials were made of a mixture of equal parts of 5% solutions of the two acids at room temperature for 1, 2, 3, 5, and 10 minutes. All tests gave fully satisfactory results.

In experiment 12 trial was made to see whether the time in the plasma stain could be reduced and whether acid fuchsin could be omitted from the Biebrich scarlet modification. Mordanting was carried on for 1 minute at room temperature with the 5% phosphomolybdotungstic mixture. Plasma stains were applied 1, 2, 3 and 5 minutes, using 1% Biebrich scarlet in 1% acetic acid alone as well as the mixture of Biebrich scarlet and acid fuchsin (9:1).

TABLE 1. DYES USED

Name	C.I. No.	Schultz No.	Manufacturer	Lot No.
"Ponceau de xylidene"			Grübler	1.37
Ponceau 2R	79		National Anilin	7895
Bordeaux red	88	112	National Anilin	4988
Chromotrope 2R	29		National Anilin	8449
Crocein	252 or 277?		Old sample	
Biebrich scarlet WS	280	247	Biosol Products	131
Azofuchsin	30		National Anilin	7788
Azofuchsin B	66		National Anilin	3210
*Azofuchsin 4G	29 a*		National Anilin	8449
Azofuchsin 6B	57		National Anilin	3210
Azofuchsin 6B, GN or S	154		National Anilin	3210
Azofuchsin G	153		Hartman Leddon	(1938)
Azofuchsin 3B	54		Hartman Leddon	(1938)
Eosin Y	768		National Anilin	Cert. NE11
Methyl eosin	769?		Grübler	Old
Erythrosin	773?		Grübler	Old
Phloxine (73%)	774		Hartman Leddon	Recent
Acid fuchsin (60%)	692	524	Hartman Leddon	Recent
Methyl blue	706		National Anilin	7624
Orange G	27		Grübler	12.37
Nitrazine yellow			(Squibbs)†	Recent
Chrysoidin (acid dye)	Not C.I. No. 20, 21, or 60		Providence Chemical	About 1930
Fast green FCF			Coleman and Bell	Recent
Anilin blue WS	707		Hartman Leddon	Recent

*This is listed in the Colour Index as similar to chromotrope 2R, C.I. No. 29.

†The lot actually used was obtained from another source and under a different name; but the company furnishing it to the writer does not put it on the market any more. A sample subsequently obtained from Squibbs labelled "nitrazine" was found to be less concentrated and had to be employed in 2% instead of 1% solution. In this paper the name "nitrazine yellow" is employed for the dye, as it was first described under that name (Wenker, 1934).

Immersion for 2 minutes in plasma stain was found fully adequate, one minute not quite enough. We considered Biebrich scarlet alone to give a better picture than the acid fuchsin mixture.

In experiment 13 trial was made to see whether the time in the

fast green fiber stain and in the acetic acid differentiation could be reduced. The timing was as follows: 2 minutes in Biebrich scarlet (alone), 1 minute at room temperature in the 5% phosphomolybdotungstic mixture and 1, 2, 3 and 5 minutes, respectively in the fiber stain with the following 1% acetic differentiation applied 1 and 2 minutes for each variant of the fiber stain. Staining for two minutes in the acetic fast green was adequate, one minute not quite adequate, and one minute differentiation in 1% acetic acid was enough.

Experiment 14 was designed to test the possibility of further reducing the mordanting time in the first mordant. Both saturated aqueous picric acid and saturated aqueous mercuric chloride, which had previously given excellent results as a mordant (Lillie, 1938), were tried at 58° C. for 2, 3, 5 and 10 minutes. The remaining steps were as in experiment 13, with 2 minutes in fast green and 1 minute in 1% acetic acid. The results were essentially identical thruout, indicating that 2 minutes at 58° C. in either of these primary mordants would be adequate. In experiment 15 the same 2 first mordants were used for 2 minutes at 58° C., and trial was made to reduce the washing interval. The balance of the procedure was as in experiment 14. Sections were washed after the first mordants for 1, 2, 3, 5, 10 or 15 minutes or briefly rinsed. A 3-minute washing was required to remove all of the visible yellow color after picric acid mordanting. No difference, however, was seen in the staining with either mordant, regardless of the length of washing.

In experiment 16 the 3-minute washing interval was adopted, the rest of the procedure remaining as before, and trial was made of saturated alcoholic picric acid (about 6%, as compared with about 1.25% for aqueous), saturated aqueous mercuric chloride and 20% alcoholic mercuric chloride as first mordants. These were tried severally for 1, 2, 3, 5 and 10 minutes at room temperature in comparison with saturated aqueous picric acid and mercuric chloride solutions for 2 minutes at 58° C. At room temperature aqueous mercuric chloride gave excellent results at 5 and 10 minutes, good at 3 minutes and irregular with poor staining of muscle at 1 and 2 minutes. Alcoholic mercuric chloride dried on the slides during the brief interval of transfer to running water and results of subsequent staining were spotty and irregular. Mordanting for 1, 3 and 10 minutes gave good results, but for 2 and 5 minutes poor, with muscle almost unstained. Saturated alcoholic picric acid gave satisfactory staining results at all mordanting durations.

In experiment 17 an attempt was made to introduce another color to render the plasma staining more differential. Instead of mixing

the dyes, it was decided to try sequence staining, with or without an intervening mordant. Two dyes were used, orange G and nitrazine yellow, a brown plasma stain, in addition to the Biebrich scarlet. 1% solutions of each were made in 1% acetic acid. Tests were made with each of these dyes, staining for 2 minutes before or after a 2-minute Biebrich scarlet stain, with or without an intervening 1-minute mordanting in 5% phosphomolybdotungstic mixture. When no intervening mordant was used, the effect obtained was the same as from Biebrich scarlet alone. When the intervening mordant was used the effect was that of the first dye alone, whether Biebrich scarlet, orange G or nitrazine yellow without trace of the color expected from the second dye. The procedure was otherwise that of experiment 16 with mordanting for 1 minute in saturated alcoholic picric acid.

Continued use showed some irregularity in plasma staining with 1 minute in the first mordant, so it seemed advisable to increase this to 2 minutes. Thus the modified procedure arrived at from the foregoing experiments is as follows:

1. Bring paraffin sections thru xylol and alcohol into saturated alcoholic picric acid for 2 minutes.
2. Wash 3 minutes in running water.
3. Stain 6 minutes in Weigert's acid iron chloride hematoxylin.
4. Rinse in water.
5. Stain 2 minutes in 1% Biebrich scarlet in 1% aqueous acetic acid.
6. Rinse in water.
7. Mordant 1 minute in a mixture of equal parts of 5% solutions of phosphomolybdic and phosphotungstic acids.
8. Stain 2 minutes in 2.5% fast green FCF in 2.5% aqueous acetic acid.
9. Differentiate 1 minute in 1% aqueous acetic acid.
10. Carry thru alcohol, acetone, acetone+xylene, and xylene (two changes) into salicylic acid balsam.

Longer staining (e.g. 3 or 4 minutes) with Biebrich scarlet is necessary when the following fiber stain is anilin blue or methyl blue.

Conclusions

1. A number of dyes may be substituted for imported "ponceau de xylidene" in Masson's ponceau acid fuchsin mixture. Among the best are ponceau 2R (C.I. No. 79), azofuchsin 3B (C.I. No. 54), nitrazine yellow, and Biebrich scarlet (C.I. No. 280). We prefer the last to any dye tried thus far. Nitrazine yellow is very good when a

brown plasma stain is desired. Biebrich scarlet makes as satisfactory a plasma stain without addition of acid fuchsin.

2. A mixture of equal parts of phosphomolybdic and phosphotungstic acid solutions is much superior, as a second mordant, to either used alone. When 5% solutions are used, the time required is 1 minute at room temperature.

3. With the fast green modification, the times in the plasma and fiber stains can be reduced to 2 minutes each. With anilin blue a 4-minute plasma stain seems indicated. One-minute differentiation in 1% acetic acid after the fiber stain appears to be adequate.

4. Primary mordanting may be reduced to 5 minutes in saturated aqueous mercuric chloride solution or to 2 minutes in a saturated alcoholic solution of picric acid, both at room temperature. Washing 3 minutes in running water after the mordant seems to be adequate.

The writer is indebted to Scientific Aide Milton Gusack for technical assistance in carrying out the experimental work herein reported.

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THE USE OF SUDAN BLACK B AS A BACTERIAL FAT STAIN

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ABSTRACT.—**Sudan black B was introduced as a specific fat stain for the detection of lipids in tissue sections by L. Lison in 1934. Saturated solutions of Sudan black B in 70% alcohol or in ethylene glycol stain the fat bodies of bacteria a deep blue-black color, and this dye is recommended as superior to the other Sudans.**

The method used in staining the bacteria was to suspend a loopful of the cells in a drop of the stain solution and to prepare flat wet mounts. The organisms giving positive fat tests with Sudan black B included *Bacillus cereus, Bacillus mycoides, Azotobacter beijerinckii, Rhizobium leguminosarum, Mycobacterium avium, Mycobacterium leprae, Oospora lactis, Bacillus tumescens,* **water spirilla, and fungi.**

INTRODUCTION

The use of the Sudans as fat stains dates back to 1896, when L. Daddi introduced Sudan III as a specific stain for fat. L. Michaelis, in 1901, reported on his synthesis of Sudan IV, which had similar staining properties and was related to Sudan III. French (1926) reported oil red O, (*Syn.*, Sudan II) as the most brilliant of the Sudan fat stains.

The above mentioned Sudans have long been recognized as the stains of choice when testing cells for fat. Recently, however, Sudan black B has been proposed as having staining properties equal to the other Sudans. It was introduced by Lison (1934) as a specific fat stain for the detection of lipids in tissue sections, and is a dyestuff of the phenyl-azo-naphthyl-azo-naphthyl type. Recently, Leach (1938) proposed 50% diacetin and distilled water as an improved solvent for Sudan black B, and also presented a method of using it to stain lipids in sections of intestinal epithelium.

Since the stains of the Sudan series readily stain cell fats, an investigation was conducted to determine the ability of Sudan black B to stain the fats of bacteria and to find a more suitable solvent for the dye, other than those already proposed, which would more readily adapt itself for the staining of lipids in bacteria.

The method of staining the fats of bacteria is to suspend the organisms in the dye and prepare flat wet mounts. For this procedure

it is of value to have a stain which will readily suspend the bacteria in question and not cause precipitates or plasmolysis of the cells due to evaporation of the dye solvent. The solvents found to meet these requirements are 70% alcohol, 50% acetone and water, 50% dioxan and water, and ethylene glycol.

Materials and Methods

The Sudan black B used during this study was furnished by the National Aniline and Chemical Company, New York City. The staining solution was prepared by dissolving 0.25 g. of the dry Sudan black B powder in 100 ml. of 70% alcohol at room temperature. Solution of the dye takes place almost immediately. The staining solution as described by Leach was prepared by adding excess of the dye to equal amounts of diacetin and distilled water followed by two days incubation at 55° C. and filtering before using.

Saturated solutions of Sudan black B in 50% acetone and distilled water, 50% dioxan and distilled water, and ethylene glycol[1] were prepared. For best results it is advisable to let the solutions stand for several days since the acetone and dioxan require considerable time for solution to take place.

The method used in staining the bacteria was to suspend a loopful of the cells in a drop of the stain solution and prepare flat wet mounts.

The organisms tested for fat during this investigation included *Bacillus cereus*, *Bacillus mycoides*, *Bacillus tumescens*, *Azotobacter beijerinckii*, *Rhizobium leguminosarum*, *Mycobacterium avium*, *Mycobacterium leprae* (strain isolated by J. R. Kriz), *Oospora lactis*, water spirilla, and fungi. (These organisms were obtained from the stock cultures of the University of Texas at Austin.)

The *Rhizobium* and *Azotobacter* cultures were grown on yeast extract mannitol agar and nitrogen-free mannitol agar respectively. The fungi studied were isolated from the air by exposing glucose agar plates for several minutes. The other test organisms were grown on 6% glycerin infusion agar and carbohydrate infusion agar.

The cultures were studied at various periods during their growth and the presence of fat bodies within them was detected by the use of Sudan black B. All positive fat tests were confirmed by the fat stains of Eisenberg (1909), by staining with Sudan II, III, and IV, by negative fat stains, and by unstained vital preparations.

[1]The ethylene glycol used as a dye solvent should have a boiling point of 195–7° C., since if less pure it will not completely dissolve Sudan black B.

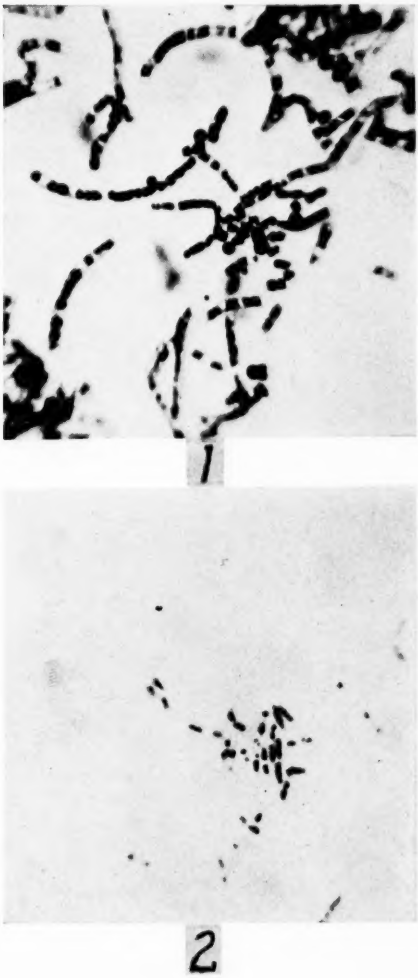

PLATE I.

Fig. 1. *Bacillus cereus* from a 48-hour culture on 6% glycerin agar, showing fat bodies stained with Sudan black B dissolved in 70% alcohol.

Fig. 2. *Bacillus tumescens* from a 32-hour culture on 6% glycerin agar, showing fat bodies stained with Sudan black B dissolved in 70% alcohol.

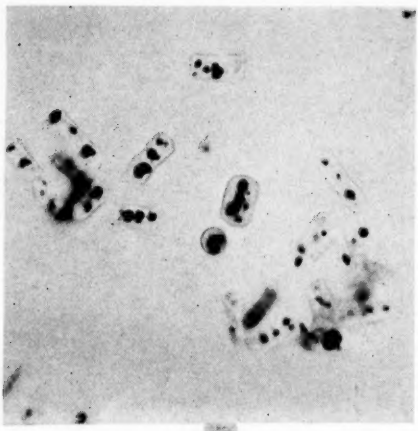

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PLATE II.

Fig. 3. *Oospora lactis* from a 48-hour culture on 6% gylcerin agar, showing fat bodies stained with Sudan black B dissolved in 70% alcohol.

Fig. 4. Water spirillum from a 12-day culture on 6% glycerin agar, showing fat bodies stained with Sudan black B dissolved in ethylene glycol.

Observations and Conclusions

All the bacteria used in this study gave positive results when tested for fat by the use of Sudan black B. Fat bodies could be detected in all the organisms within 24–48 hours; however, the *Mycobacteria* gave better results if tested for fat after 7–14 days incubation. The fat droplets appear as blue-black bodies in a clear colorless cytoplasm. (Figs. 1, 2, 3, and 4). At times the organisms may assume a beady or chain-like appearance, since only the stained fat bodies are visible (Figs. 1, 2, and 4) while the cell membrane is indistinct or not visible at all.

The fat bodies stain a deep blue-black color when stained with the 70% alcohol staining solution; they assume a blue color when ethylene glycol, 50% dioxan and distilled water, and 50% diacetin and distilled water are used as the dye solvents. When Sudan black B is added to 50% acetone and distilled water, a red solution is formed which stains the fat bodies of the bacteria red; this is due to the fact that many organic dyestuffs have an entirely different color in organic solvents and only give their true color when the solvent is evaporated.

The most brilliant fat tests were observed when the bacteria were stained with the dye solutions using either 70% alcohol or ethylene glycol as the solvent. Ethylene glycol seems to be especially suitable as a dye solvent for Sudan black B, since it does not tend to evaporate when making vital mounts, and in addition has the property of taking on moisture, thus permitting preparations to remain in excellent condition for several days. The other solvents gave less striking results, altho the fat bodies were stained a bright blue color. The stain solution as recommended by Leach did not give very satisfactory results, as the fat bodies stained a faint blue and at times did not stain at all. Forty per cent alcohol, which has been recommended as a solvent for Sudan black B, proved to be unsatisfactory because insufficient dye was dissolved.

Sudan black B in 70% alcohol has retained its staining ability for over six months and does not seem to have deteriorated in any way. Dye solutions using ethylene glycol, 50% dioxan and distilled water, and 50% acetone and distilled water as solvents, also have kept their staining abilities over long periods of time. Frequently a dark precipitate is observed in the wet-mount preparations; however, since it is usually in the background and beyond the focal level of the cells, it is of no objectionable consequence. Therefore, it can be concluded that the dye solvents tried in this study do not tend to deteriorate upon aging or to cause undesirable precipitates or to give poor staining reactions.

From the observations made during this study, Sudan black B is to be preferred to Sudan II, III or IV as a bacterial fat stain. The solubility of the Sudan black B in the various solvents tried, as well as the slight solubility of the other Sudans in similar solvents, is a matter of practical importance. The microscopical picture obtained when using Sudan black B as compared to the other Sudans would indicate that Sudan black B presents a less confusing picture and is equally specific for fat substances.

Summary

Sudan black B dissolved in 70% alcohol or ethylene glycol stains the fat bodies of bacteria a deep blue-black color, and is recommended as superior to the other Sudans.

The author wishes to express his appreciation to Dr. I. M. Lewis, Department of Botany and Bacteriology, University of Texas, for his helpful advice and criticism during this work.

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A SIMPLE TECHNIC FOR IN TOTO STAINING OF TARSAL AND SEBACEOUS GLANDS

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In toto or bulk staining with Sudan IV as used by Herxheimer is an efficacious method for delineating tarsal and sebaceous glands. Altho Sudan III and Sudan IV have long been used for staining fatty substances in general, the lack of references to the application of these stains for purposes referred to above indicates that the demonstration of entire tarsal and sebaceous glands is not in general use. Fatty substances in general stain more deeply with Herxheimer's stain than with Sudan III.

The eyelids and skin removed from animals (dog, ox, cat and human cadavers) were fixed in 10% formalin for 24 hours or longer and then run thru 50% alcohol (ethyl) into 70% alcohol. Care should be taken to wash out the fixing fluid thoroly with several changes of 70% alcohol. This will avoid the formation of a precipitate after the tissue is placed in the stain.

Cadavers, when received at the Indiana University anatomical laboratories, have already been injected with a preservative, the ingredients of which are unknown. The eyelids and skin removed from them for purposes designated above are fixed in 10% formalin before they are finally injected with a mixture of equal parts of alcohol, glycerin and phenol.

To delineate the tarsal glands clearly, the tissues covering the tarsus should be removed. This can be easily done by submerging the eyelid in 70% alcohol in a large Petri dish, the bottom of which has been coated with a layer of paraffin about 5 mm. thick, and by pinning the lateral and medial margins of the eyelids to the paraffin. Care should be taken not to insert the pins thru the tarsus. When the eyelid is thus anchored, the skin, orbicularis oculi muscle and the palpebral conjunctiva can be removed easily from the tarsus with a small pair of curved scissors and a pair of fine forceps. The use of binocular magnifying lenses with a long working distance is an aid in removing the excess tissue. A binocular loupe is very satisfactory to use with this type of work.

If it is also desirable to display the large sebaceous glands that open into the hair follicles of the eyelashes, the epidermal layer of the skin near the margin of the eyelids should be removed.

For the delineation of the sebaceous glands, free-hand vertical sections 1 to 2 mm. thick are made from any region of the integument where these glands are present (scalp, skin of general body surface). The sections are more easily made from fixed than from fresh material. Whole mounts of skin 12 mm. square or larger also make interesting preparations.

The formula for Herxheimer's stain[1] is as follows:

70 parts of absolute alcohol (ethyl),
20 parts of a 10% solution of sodium hydroxide,
10 parts of distilled water,
Sudan IV to saturation.[2]

The procedure for staining is as follows:

1. Transfer tissue from 70% alcohol to Herxheimer's stain. Staining is accomplished in 12–24 hours or in less time if the pieces of tissue are thin.

2. Wash out excess stain with repeated changes of 70% alcohol until the glands are sharply delineated.

3. Transfer to glycerin. The tissues surrounding the glands become semitransparent. The preparation may be kept permanently in glycerin, or may be mounted in glycerin jelly. Of the several formulae for glycerin jelly, Brandt's formula[3] was used: melted gelatin 1 part; glycerin 1½ parts; and a few drops of carbolic acid.

Tarsal and sebaceous glands stained by this method seven years ago have not faded and make beautiful whole mount preparations to supplement the study of these glands in ordinary microscopic preparations.

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[1]Enzyklopädie der Mikroskopischen Technik, Dritte Auflage, Bd. 1, 729–30. Urban und Schwarzenberg, Berlin-Wien.

[2]The dye used for staining the majority of the specimens was labeled "Scharlach R" and purchased a good many years ago from the Harmer Laboratories of Lansdowne, Pa. For some of the recent specimens a lot marked "Scarlet R" obtained from the Coleman and Bell Co. was employed. It was subsequently pointed out by the Chairman of the Biological Stain Commission that altho the name "Scarlet R" denotes an entirely different type of dye to the dye industry it is often incorrectly employed in the medical literature as a synonym of Sudan IV. He accordingly furnished a sample of a dye sold under the latter name by the National Aniline and Chemical Co. and certified by the Stain Commission; this sample was tried and gave equally good results.

[3]Lee, Bolles. 1937. The Microtomist's Vade-mecum, 10th ed., edited by J. B. Gatenby and T. S. Painter. Churchill, London. (See p. 223).

LABORATORY HINTS
FROM THE LITERATURE

A DEPARTMENT DEVOTED TO ABSTRACTS OF BOOKS AND PAPERS FROM OTHER JOURNALS DEALING WITH STAINS AND MICROSCOPIC TECHNIC IN GENERAL

The abstracts given here are intended primarily for laboratory use; consequently the technic in each instance is given in as much detail as possible.

J. A. de Tomasi — *Abstract Editor*

BOOK REVIEWS

BECKER, ELERY R. and ROUDABUSH, ROBERT L. **Brief Directions in Histological Technique.** 6 x 8½ in., 80 pp. Paper, with spiral binding. Collegiate Press, Inc., Ames, Iowa. 1939. $1.00.

According to the authors' statement in the preface, "This manual was prepared especially for use by classes in histological technique in the Zoology and Entomology Department at Iowa State College." Altho the book makes no pretention of having general applicability, it may prove useful in some other laboratories where an inexpensive handbook of this sort is desired. The writers give detailed directions for one standard histological procedure (Zenker-paraffin with Delafield's hematoxylin), and follow this with alternate procedures for the various steps involved. The chapter dealing with the standard procedure is about 18 pages long; and this is followed by five shorter chapters dealing with the alternate procedures for fixation, dehydrating, imbedding, staining, and mounting, respectively. A final chapter deals with special methods for tissue or microörganisms to which the more general histological technic is not applicable.—*H. J. Conn.*

MICROSCOPE AND OTHER APPARATUS

MELEZER, N., and VENKEI-WLASSICS, T. **Die Quecksilberhochdrucklampe als Lichtquelle für Fluoreszenzmikroskopie und Mikrophotographie.** *Zts. wiss. Mikr.*, **56**, 202–10. 1939.

The author suggests the use of a new tungsten-mercury-vapor lamp developed by the Simons Company in place of the expensive low voltage ultraviolet quartz lamps or the unsatisfactory ultraviolet arc lamp using iron electrodes. These lamps, designed on the general plan of the ordinary tungsten incandescent lamp with Edison base, burn evenly emitting a constant source of light having a wave length between 310–390 mμ. They operate on 220 volts A. C.—*J. M. Thuringer.*

SCOTT, G., and PACKER, D. M. **The localization of minerals in animal tissues by the electron microscope.** *Science*, **89**, 227–8. 1939.

Partial absorption of an electron beam is known to give rise to profile pictures of bacteria, or epidermal cells. Such a beam can also be used to cause thermoionic excitation of electrons on metallic surfaces. This principle is applied here in an attempt to localize minerals in sections of animal tissues. Sections are ashed at greatly reduced pressure on the surface of a cathode coated with Ba and Sr in the electron microscope. As most of the inorganic elements in tissues are excited to thermoionic emission at relatively specific tempertaures, a differentiation and localization of at least Mg and Ca is thus made possible. A picture of cellular structures in striated muscle, gastric mucous membrane, and nerve tissue can then be obtained.—*J. A. de Tomasi.*

ZELLER, A. **Ein neues Kanadabalsamfläschchen.** *Zts. wiss. Mikr.*, **56**, 211. 1939.

A simple and practical balsam bottle is described having a large ground glass cover. An ingenious perforated groove around the neck of the bottle, beneath the cover, permits a return flow of any balsam spilled.—*J. M. Thuringer.*

MICROTECHNIC IN GENERAL

LANDAU, E. **Appareil permittant la déshydration et l'enrobage d'une pièce dans le vide, sans recourir aux substances chimiques.** *Bull. d'Histol. Appl.*, **16**, 13–8. 1939.

The author's apparatus for fixing fresh tissues by drying while freezing is described. With this apparatus, fresh, unfixed tissue can be dehydrated without employing any of the usual chemical reagents and with no shrinkage of the tissue. Dehydration is accomplished by a system of three pumps giving an almost complete vacuum, while a gas refrigerating system lowers the temperature to about −25°C. During this process the tissue may rest upon paraffin which can then be melted by means of a warm water bath. The tissue (still in the vacuum) is thus automatically immersed in paraffin. Infiltration and embedding are completed in 15–20 min.—*M. Noble Bates.*

MILOVIDOV, P. **Die Anwendung der Azetokarmin-Methode für die Färbung von fixierten Mikrotomschnitten.** *Zts. wiss. Mikr.*, **56**, 67–9. 1939.

The technic recommended has been employed for material fixed in each of the following fluids: Flemming, Němec IIIa, Nawaschin, Němec-Milovidov, Němec I, sublimate-acetic, alcohol, *etc.* Deparaffinized sections are covered with a few drops of Schneider's aceto-carmine and gently heated for 1–2 min. (without boiling). After cooling, they are rinsed in water and transferred thru 96% alcohol, abs. alcohol, xylene, to Canada Balsam. Best cytoplasmic staining is obtained after alc. or sublimate-acetic fixation. The best general results are obtained with Flemming's, Němec's IIIa, and Nawaschin's fluid. This method has the advantages of rapidity and permanency.—*J. M. Thuringer.*

ROBINSON, B. G. **A note on mounting thin celloidin-sections.** *J. Roy. Micro. Soc.*, **59**, 79. 1939.

Some of the difficulties encountered when mounting thin sections of material embedded in celloidin and paraffin wax can be eliminated by the following method. Infiltrate the material in the usual way. Then immerse the trimmed block in abs. alcohol until perfectly transparent; harden in xylene and transfer to the waxes. Float ribbons of sectioned material on slides previously smeared with albumen, stretch them on the hot-plate, drain and immediately store in a closed glass vessel containing cotton-wool soaked in ether and a small dish of $CaCl_2$. The ether vapor flattens the sections and the $CaCl_2$ drys them. Sections may be stored indefinitely in such a vessel. Before the ether, alcohol and staining baths, a photographic squeegee-roller may be lightly rolled over the slides; the slides are then able to withstand immersion in watery reagents for several days without the loss of a single section from the series.—*H. E. Finley.*

SUTER, HANS. **Über die Eignung der Schlierenmethode zur Messung osmotischer Zustandsgrössen.** *Protoplasma*, **31**, (3), 421–53. 1938.

The author presents in detail the application to biological material of the Töpler-Schlieren method (Töpler, A., Ann. d. Phys., **4**, 838. 1904) which depends upon the diffusion of substances of different refractive indices in a solution. A leaf of *Sempervivum tectorum* is carefully removed and the cut base of the leaf sealed with paraffin after the epidermis has been carefully stripped off. The leaf is then placed in water or in a sugar solution, and by appropriate illumination, striae or the so-called Töpler-Schlieren can be detected in accordance with diffusion gradients and density changes in the medium over the exposed cell sap.—*Robert Chambers.*

WOLF, JAN. **Über die Herstellung mikroskopischen Präparate der Obefläche verschiedener Objecte mit Hilfe der Adhäsionsmethode.** *Zts. wiss. Mikr.* **56**, 181–201. 1939.

The author describes the production of microscopic preparations of various surfaces with the aid of the "adhesion" method. This interesting new process utilizes celloidin casts of surfaces which may be examined by transmitted light because the images develop at the point of contact of air with the differently refracting celloidin medium. To obtain a cast of the surface epithelium of the

epidermis, the following procedure is used: (1) Paint the dry surface with a thin coat of celloidin and allow to dry from ½ to 1 min. (2) Place a piece of transparent cellophane tape over this area, avoiding the formation of air bubbles between the two layers. (3) Remove tape and celloidin with a quick movement. (4) Place on coverglass (adhesive surface down) and mount on glass slide. It may be noted that it is the under or inner surface of the cast that becomes visible on examination. The results are very interesting and instructive. The method permits modifications, *i.e.*, surface cells may be removed *in toto* and examined against the celloidin matrix or removed with adhesive tape alone and transferred to slides for examination in transmitted light with or without staining.

The method promises to become useful in histology, zoology, botany, pharmacognosy, and criminology.—*J. M. Thuringer.*

DYES AND THEIR BIOLOGICAL USES

COLLANDER, R., and VIRTANEN, E. **Die Undurchlässigkeit pflanzlicher Protoplasten für Sulfosäurefarbstoffe.** *Protoplasma*, **31** (4), 499–507. 1938.

The authors champion the findings of Plowe (Protoplasma, **12**, 196. 1931) and of Chambers and Kerr (J. Cell. Comp. Physiol., **2**, 105. 1932) that sulfonic acid dyes, altho highly diffusable, do not penetrate living cells. The present authors used varying concentrations of light green, acid fuchsin, and orange G in aq. media buffered with citrate and phosphate to pH 1.9 to 8.5. The material they used was the mycelium of *Aspergillus niger* which had been claimed by Bünning (Flora N. S., **31**, 87. 1936) to take up these dyes. They also found that orange G and cyanol are not taken up by *Tolypellopsis stelligera* even from concentrated solutions.—*Robert Chambers.*

HÖBER, R., and BRISCOE, P. M. **Correlation between secretion of dyestuffs by the kidney and molecular structure of these dyes.** *Proc. Soc. Exp. Biol. & Med.*, **41**, 624–6. 1939.

Some 30 mono-azo-sulfonic acid dyes were tested by perfusing isolated frogs' hearts with Ringer's solution and then with 0.0005% dye solutions. The position of the sulfonate group appeared to be the controlling factor in secretion or failure to secrete the dye. The suggestion is offered that, if sulfonate groups are attached to only one-half of the molecules, the sulfonated half may be hydrophilic and the other half may be hydrophobic and organophilic, permitting anchoring between the cell and surroundings as the first step in penetration.—*M. S. Marshall.*

KEMPTON, R. T. **Differences in the elimination of neutral red and phenol red by the frog kidney.** *J. Cellular and Comp. Physiol.*, **14**, 73. 1939.

The excretion of phenol red by the normal and narcotized frog kidney has been studied in relation to changes in pH of the urine. It was found that in contrast to that of neutral red, the phenol red elimination was unaffected by changes in urinary pH. As with neutral red, phenol red elimination was stopped in the narcotized kidney. This inhibition of phenol red elimination is explained by an interference with cell activity, whereas that of neutral red is apparently caused by the alkalinizing of the urine subsequent to the stopping of acid excretion.—*L. Farber.*

KRAJIAN, ARAM A. **A new frozen section method for the preparation of permanent frozen sections of loose texture tissues.** *Amer. J. Clin. Path., Tech. Suppl.*, **3**, 189–92. 1939.

Add 2 cc. of formalin to 50 cc. of fresh uncontaminated pooled human or animal serum. This keeps several weeks at room temp. Fix loose texture tissues in 10% formalin 24 hr. or longer. Trim and wash to remove formalin. Put in serum and keep in a warm place (e.g., top of paraffin oven) over night. Cover any floating sections with absorbent cotton. Decant excess of serum and add dioxan. Stand 3–5 hr. or until completely coagulated. Freeze, cut, stain and mount as usual. Preserve blocks in 10% formalin; dioxan causes shrinkage. For emergency, put thin blocks of formalin fixed tissue in serum in a paraffin oven at 56° C. 1–2 hr. Coagulate with dioxan 1 hr.—*George H. Chapman.*

YOE, JOHN H., and BOYD, GEORGE R. **Patent blue V as a pH and redox indicator.** *Ind. Eng. Chem., Anal. Ed.*, **11**, 492–3. 1939.

An aq. solution of patent blue V may be used as an indicator for the colorimetric determination of pH over the interval 0.8 to 3.0. The colors range from yellow thru green to blue and are stable for periods up to 5 days, after which a very slight fading may be detected. The change in color is pronounced and the pH of a solution within the range given above, can be determined to 0.1 of a pH-unit by comparison with standards made up at intervals of 0.2 pH. The same dye sample must be used for both standards and unknowns because solutions of the dye from different sources often exhibit slightly different colors at a particular pH-value.

Patent blue V can also be used as an oxidation-reduction indicator in certain volumetric methods. Altho it cannot be used with $K_2Cr_2O_7$ or in the presence of HCl, it can be used with $KMnO_4$ or $CeSO_4$ if all HCl has been removed. The oxidation-reduction potential of the purified indicator was 0.78 volt. This value corresponds almost exactly to the potential at the equivalence point in the ferrous-ferric system—namely, 0.77 volt.—*A. P. Bradshaw.*

ANIMAL MICROTECHNIC

BAYLEY, J. H. **Differential staining methods for formalin-fixed human pituitary gland.** *J. Path. & Bact.*, **49**, 261–3. 1939.

The technic described for differential staining of the granule cells in the anterior lobe of the pituitary has the advantages of being applicable after ordinary formol fixation, of permitting good staining of basophil granules, and of eliminating mitochondrial staining. The following are considered the most useful and permanent methods.

General procedure: Fix whole gland in 10% neutral formol-saline for 12 hr., bisect and replace in formol for a further 24 hr. Prepare paraffin sections as usual; xylene, 2 min.; abs. alcohol, 1 min.; sat. aq. $HgCl_2$, 2 min. (to improve later staining); Lugol's iodine, 2 min. To avoid staining mitochondria, bring sections to dist. water; 0.5% $KMnO_4$, 1 min.; 5% oxalic acid, 2 min.; rinse well with tap-water. Stain 2 min. with hot but not boiling 1% aq. acid fuchsin (Grübler). Rinse in dist. water. Stain 1–3 min. in the following solution: Acid violet (Revector, supplied by Vector, Ltd.), 1 g.; orange G, 0.3 g.; dist. water, 200 cc. Rinse in dist. water. If overstained in the acid violet mixture, differentiate with 80% alcohol or in extreme cases with Lugol's iodine. Rinse; dehydrate in abs. alcohol, 2 min.; xylene, 2 min.; mount in balsam. Eosinophils are crimson, basophils dark blue, chromophobe cells grey, erythrocytes crimson, and connective tissue grey.

For connective tissue proceed as before until staining. Stain 5 min. in hot but not boiling 1% aq. acid fuchsin; rinse; stain 1–3 min. in the following: acid violet (Revector), 1 g.; orange G, 0.5 g.; acid green (Revector), 1 g.; dist. water, 200 cc. Rinse; differentiate with Lugol's iodine (5 sec.-1 min.); abs. alcohol, 2 min.; xylene, 2 min.; Canada balsam. Muscle is dark red, fibrous tissue dark blue.—*S. H. Hutner.*

CARTER, W. **The use of prontosil as a vital dye for insects and plants.** *Science*, **90**, 394. 1939.

Corn seedlings, with or without roots, take up neoprontosil (a red dye) very rapidly. Their leaves show red streaks within even a few seconds, and insects, like the corn leafhopper, will show presence of the dye in their tissues after feeding on dye-saturated leaves for a day or two.—*J. A. de Tomasi.*

DONAGGIO, A. **Dimostrazione dell' esistenza di una lesione organica reversibile nell' azione degli anestetici sulle fibre nervose centrali e periferiche.** *Arch. exp. Zellforschung*, **22**, 171–80. 1939.

The following method is said to leave only the anesthetized nervous tissue stained: Fix 36 hr. in Zenker's fluid; rinse in dist. water; place in amber colored aq. iodine (a few drops of tincture of iodine in dist. water) for 48 hr.; rinse in dist. water; place in 2.5% $K_2Cr_2O_7$ 4–5 min. at 37 C.; dehydrate in a series of alcohols; embed in celloidin; section at 20μ; immerse sections in Lugol's solution (5g. KI,

0.5g. I_2, 5g. water, 45g. 90% alcohol) to eliminate traces of ppt.; wash in 95% alcohol for 1 hr.; pass thru 70% and 50% alcohol to dist. water, a few min. in each; stain in stannic hematoxylin (add 1% aq. hematoxylin at least a week old to an equal amount of 20% aq. $SnCl_4$) for six hr.; partially decolorize by Pal's method (0.1% $KMnO_4$, followed by a mixture of 1 g. oxalic acid, and 1 g. K_2SO_3, or preferably $Na_2S_2O_3$, in 200 cc. dist. water); examine under low power until the color is removed from the normal tissue.—*V. W. Kavanagh.*

GOMORI, G. **The effect of certain factors on the results of silver impregnation for reticulum fibers.** *Amer. J. Path.*, **15**, 493–5. 1939.

The impregnation of reticulum fibers by ammoniated alkaline silver reagent (Gomori, Amer. J. Path., **13**, 993–1002. 1937) is only slightly affected by fixation. The following fixing fluids were used with organs from man, dog and guinea pig: alcohol, Carnoy's fluid, formalin-alcohol (1:5), formalin (1:10, presumably aq., but not stated), Bouin's, Orth's, Zenker's, Stieve's, and Zenker-formalin (9:1). Carnoy's gave deep black staining of reticulum fibers with gray nuclei and almost unstained cytoplasm. Cytoplasm, but not nuclei, were stained after Bouin's; other fixatives gave results similar to formalin. Exhaustive oxidation of the sections by 2 or 3 treatments with acid $KMnO_4$ (0.5% H_2SO_4 added to 0.5–1% $KMnO_4$) alternated with decolorization by a 1–3% solution of $K_2S_2O_5$ intensified the differentiation between reticulum fibers and other tissue elements. Length of time of fixation in formalin from 12 hr. to several months did not affect staining, neither did decalcification with either nitric or sulfosalicylic acid. Thin sections, 8μ or less, are preferred.—*H. A. Davenport.*

JACOBSON, W. **The argentaffine cells and pernicious anemia.** *J. Path. & Bact.*, **49**, 1–19. 1939.

The granules of the argentaffine cells of the gastro-intestinal tract may be stained by taking advantage of their ability to reduce silver salts and to couple with diazotizing agents. For both methods tissue may be fixed in 10% formol-saline or 10% neutral formol, dehydrated with alcohol, cleared in cedarwood oil or in methyl benzoate plus 2% celloidin, and imbedded in paraffin.

The following modified Masson-Hamperl silver staining technic was used: Wash deparaffinized sections in glass-dist. water (10 min. is enough if two changes of water are used); place 12–24 hr. in Fontana's solution (prepare by adding NH_4OH to 5% $AgNO_3$ until the precipitate is dissolved, then add more $AgNO_3$ drop by drop until the fluid shows a slight persistent opalescence; glass-dist. water must be used); wash slides 1 min. in glass-dist. water; 5% $Na_2S_2O_3$, 1 min.; tapwater, 10 min. Only the granules of the argentaffine cells appear black. Nuclei may be counterstained with carmalum and the sections permanently mounted in Canada balsam.

An alternative quicker diazo method is as follows: prepare diazotizing solution by dissolving small amount of *p*-nitro-methoxybenzene diazotate (Kernechtrotsalz B) in dist. water to produce a light yellow solution. Alkalinize with a small amount Li_2CO_3. About ½ min. later pH 10–11 is reached, the color changing to dark orange-yellow. Take sections from dist. water; immerse in stain, 30–40 sec.; wash 1 min. in dist. water. Granules of argentaffine cells are dark red against a yellow background. Nuclei can be counterstained with hemalum.—*S. H. Hutner.*

KATÔ, HIDEHARU. **Über den Einfluss der Fixierung auf das Hirngewicht.** *Folia Anat. Jap.* **17**, 237–97. 1939.

This study on the influence of fixation on brain weight was based on the examination of an extensive series of mouse, rat, cat, dog, monkey, and human brains fixed respectively, in Müller's, Helly's, Orth's, Zenker's, Ciaccio's, Carnoy's fluids, in alcohol and in 10% or 20% formalin.

It was found that Müller's, Helly's, Zenker's fluids and formalin produced an increase in weight while Carnoy and watery alc. solutions caused the brains to lose weight. Ciaccio's fluid did not influence the weight appreciably one way or another. Formalin fixation of human brains produced an average increase in weight of 10%–12% in 3–4 days, whereupon the weight remained constant for about 3 months, then diminished slowly to an average of 1.9% above their original weight after 24 months fixation.

The author stressed that brains of various animals reacted in a definite individual manner. The results are given in 37 tables and 7 graphs.—*J. M. Thuringer.*

LEPLAT, G. **Des avantages de la glycérine dans la déshydration des tissus conjunctifs et des os, avant l'enchâssement.** *Bull. d'Histol. Appl.*, **16**, 118–21. 1939.

Impregnation with glycerin is recommended to counteract the hardening and shrinking effect of the higher alcohols upon tissues which previously have been decalcified.

Good sections of the acoustic epithelium with Reissner's membrane always intact are obtained when (after decalcification with an aq. solution of HNO_3, treatment with Na_2SO_4, and washing) the labyrinth is placed for 2–3 days in each of the following: (1) a solution of 1 part glycerin to 3 parts dist. water, (2) a solution of equal parts glycerin and water, (3) pure glycerin. Transfer for 2 hr. to abs. alc. to complete dehydration and to remove the glycerin. Place in an abs.-alc.-ether mixture. Embed in celloidin.

Difficulty in obtaining serial sections of bone dehydrated in the usual manner is overcome if glycerin is employed in the process. Pieces of bone decalcified, washed, and run up to 50% alcohol are treated as follows: (1) 50% alcohol, 6–10 hr. (2) Anhyd. glycerin and 70% alcohol in equal parts, 24 hr. (3) Pure glycerin, two changes in 24 hr. (4) Abs. alc., 2 hr. (5) Clear in cedar oil and embed in paraffin.

In other tissues which are difficult to section serially (but which do not contain bone), the author finds that a certain quantity of glycerin added to the higher alcohols (70%–95%) in the dehydration process results in easier and more regular sectioning.—*M. Noble Bates.*

PERDRAU, J. R. **Ammonium molybdate as a mordant for Mann's stain and the Weigert-Pal method.** *J. Path. & Bact.*, **48**, 609–10. 1939.

For consistent Mann staining, treat material fixed in mercuric mixtures as follows. Place sections, Hg-free, in 2% ammonium molybdate overnight at 37° C. Wash thoroly in dist. water for 5–10 min.; leave in Mann's mixture overnight. Wash in dist. water and differentiate in 70% alcohol tinged with a trace of orange G, controlling the process under the low power of the microscope. Dehydrate, clear and mount. Overmordanting and overstaining is impossible.

With Mann's original staining solution the following results are obtained: Chromatin, nuclear membrane, cell membrane and connective tissue are blue; nucleoli, erythrocytes, cytoplasmic inclusions and many cell granules bright red; the rest pale pink. With other fixatives the background may be blue instead of pink.

Old bichromate-hardened frozen sections of nervous tissue that have been kept a long time in alcohol after hardening can be made to stain properly, myelin included, by mordanting in ammonium molybdate.—*S. H. Hutner.*

POURSINES, Y. **Techniques de coloration myélinique et cellulaire (type Nissl) du tissu nerveux, sur coupes à la paraffine provenant d'un même bloc.** *Bull. d'Histol. Appl.*, **16**, 128–34. 1939.

The steps of a myelin technic for paraffin sections are described. A 4–6 day ferric impregnation of the tissue by a 5% aq. solution of ammoniacal iron alum is done before embedding. (To secure a good impregnation, the formalin-fixed tissue has to be treated first with 95% alcohol followed by an ether-alcohol mixture).

Mounted sections are mordanted in 5% iron alum solution, stained with Regaud's hematoxylin, and differentiated with $KMnO_4$ and 2% iron alum. Myelin sheaths are stained dark gray or black.

Other or alternate sections cut from the same block may be stained to show Nissl bodies, or may be stained with hematoxylin and eosin, by first treating them with oxygenated water or with HCl, so as to modify the iron compounds deposited in the tissue during the ferric impregnation. Directions are given for this treatment and the staining with Unna's polychrome blue.—*M. Noble Bates.*

SCHROEDER, KURT. Eine weitere Verbesserung meiner Markscheidenfärbemethode am Gefrierschnitt. *Zts. gesam. Neurol. u. Psychiat.*, **166**, 588–93. 1939.

For staining myelin sheaths in frozen sections, the following technic is recommended:

Fix nervous tissue in formalin as usual. Cut 20–30 μ frozen sections. Mordant 24 hr. at 37° C. in a mixture 1 vol. of Müller's fluid and 2 vol. of Weigert's rapid mordant. (Composition of the rapid mordant is not given, but presumably consists of CrF_3 2.5 g., water 100 cc., heated to boiling in a covered vessel, with 5 cc. glacial acetic acid and 5 g. of copper acetate added in the order given). Wash quickly in dist. water and transfer to the staining solution made as follows. Add 3 cc. of any kind of 10% alc. hematoxylin solution to 100 cc. dist. water and boil 5 min. Cool and add 3 cc. sat. aq. soln. $LiCO_3$. Make up to 100 cc. if there is much loss in boiling. Stain sections for 12 hr. or longer at 37° C. Wash well and treat with 0.25% aq. $KMnO_4$ about ½ min. Wash twice in dist. water during the next minute and differentiate in a solution made by mixing equal parts of 1% aq. oxalic acid and 1% aq. K_2SO_3. Keep the sections in motion during differentiation and change the solution after ¼ to ½ min. Continue the differentiation until the gray matter is clear, then soak 15 min. in sat. aq. $LiCO_3$, 1 cc., plus tap water, 100 cc. Wash thoroly, dehydrate and mount in balsam. The method can be used for gelatin embedded frozen sections with the following modifications: Mordant 5 days at 37° in Müller's fluid, stain several days, use 1% $KMnO_4$, and double the concentration of the differentiator.—*H. A. Davenport.*

SPEK, J. Studien über die Polarität der Larven der Kalkschwämme. *Protoplasma*, **30** (3), 352–72. 1938.

The author used traces of dil. solutions in sea water of brilliant vital red (Grübler), Nile blue sulfate B, and brilliant cresyl violet on the blastulae of *Sycandra setosa* and *Leucandra aspersa*. Particularly with brilliant cresyl violet, he obtained striking color differences (from blue to red) in the different cells of the blastulae. These color differences he ascribed to differences in pH of the cytoplasm. His illustrations indicate that the color appears in the granular contents of the cells.—*Robert Chambers.*

SPOERRI, ROSETTE. A new material for mounting nerve tissue sections in paraffin for silver staining or restaining. *Science*, **90**, 260. 1939.

Egg albumin is not always a satisfactory fixative for nerve tissue sections. The following starch paste was found reliable: suspend 1 g. starch in 10 cc. cold water, add 20 cc. boiling water, and stir until homogeneous. Add 2 drops HCl, and boil 2–5 min. After cooling, preserve with a small crystal of thymol. Use like albumin, allowing the sections to dry on the slide 3 days at 45° C.

For Ag impregnations of sections on slides, the technic is as follows: fix material in formalin, section in paraffin, and affix to slides. Dry; pass thru xylene into water, and up thru graded alcohols to pyridine for 15 min. Put in fresh pyridine overnight. Wash 10 min. in dist. water, and impregnate 3 hr. in the dark in 5% $AgNO_3$. Transfer for 30 min. to ammoniacal $AgNO_3$ (5% $AgNO_3$, 200 cc.; 10% NaOH, 5 cc.; with NH_4OH added drop by drop until precipitate dissolves). Wash rapidly in dist. water, soak 5 min. in 10% formalin. Wash; tone in gold, or fix 2 min. in "hypo"; dehydrate; clear; and mount.—*J. A. de Tomasi.*

TERRY, R. J. A thoracic window for observation of the lung in a living animal. *Science*, **90**, 43–4. 1939.

An instrument has been devised suitable for observation of the superficial air sacs and alveoli in the cat's lung. It consists of a bronze cylinder with a small coverglass window mounted at one end, and provided at the other with a quadrilateral plate or flange. The instrument is placed in position thru an opening in one of the intercostal spaces in the thoracic wall, so that the end-plate rests against the inner surface of the wall while the window faces the lung. When the air in the pleural cavity is taken out by exhaustion, the lung surface comes to rest against the window and can be studied most conveniently. Observations are carried out with a binocular and a skin microscope. A motion picture of local phenomena has been obtained.—*J. A. de Tomasi.*

WALLART, J. **Essais de coloration de l'hypophyse.** *Bull. d'Histol. Appl.*, **16**, 149–52. 1939.

In an attempt to distinguish more clearly between the different cells of the hypophysis, the author developed a modification of existing staining methods. After preparing the sections in the usual way, they are stained as follows: Cover with Masson's ponceau-fuchsin or 1% acid fuchsin, 5 min.; wash briefly in dist. water; add 5% phosphotungstic acid, 5–8 min.; drain the surplus without washing; add Krall's anilin blue or Hollborn's standardized anilin blue, 3–5 min. with slight agitation; rinse quickly in running water; immerse in 1% acetic acid in dist. water, 30 min.; 1% acetic acid in abs. alcohol, ½–1 min.; abs. alcohol; toluene; Canada balsam.

The results are: Chromatin of the nuclei ordinarily blue, sometimes reddish or even bright red, especially in degenerating nuclei; the nucleolus most often bright red but sometimes bluish; karyoplasm sometimes colorless, sometimes bluish to deep blue; the acidophiles red; the basophiles blue; granules of the principal cells gray-blue; erythrocytes red to blue-gray.—*Jean E. Conn.*

PLANT MICROTECHNIC

BALDWIN, J. T. **Chromosomes from leaves.** *Science*, **90**, 240. 1939.

The following smear method for chromosomes from leaves is a modification of Warmke's technic (Stain Techn. **10**, 101–3, 1935): Fix young leaves in Carnoy's for 5 min. or longer; pass briefly thru 95% alcohol and conc. HCl, 1:1; carry back into Carnoy's, and after a few min. smear out in the aceto-carmin.—*J. A. de Tomasi.*

MICROÖRGANISMS

BROADHURST, JEAN and PALEY, CHARLES. **A single-dip stain for the direct examination of milk.** *J. Amer. Vet. Med. Assoc.*, **94**, 525–6. 1939.

This staining procedure is essentially that employed in the Newman technic for the direct microscopic count of bacteria in milk. It differs in the inclusion of basic fuchsin for the staining of the background material. This, in the opinion of the authors, makes for more accurate counts with less eye strain.

Preparation of Stain. Add 0.4 cc. of concentrated H_2SO_4 (measured accurately) to 54 cc. of 95% alcohol. Mix with 40 cc. of technical tetrachlorethane in a flask and heat to about 55° C. (no higher). Add the combined solution while hot to from 1.0 to 1.2 g. of methylene blue; shake until dye is dissolved. Add 8.0 cc. of a 1% solution of basic fuchsin in 95% alcohol. Mix well, cool, filter, and store in tightly stoppered bottles.

Directions for Use. Prepare milk smear as usual, by spreading 0.01 cc. of milk on a slide over an area of 1 or 2 sq. cm., as preferred. Dry the smear on a flat surface in a warm place within 5 min. When dry, dip the slide in the stain or flood the slide with the stain for about 15 sec. Drain off excess stain and dry while flat in a warm place. Wash in cold water until all the blue is washed out of the smear and it assumes a faint pink color. Dry and examine under an oil immersion objective.—*A. Zeissig.*

DELAPORTE, B. **Sur les acides nucléiques des levures et leur localisation.** *Rev. Gen. de Botanique*, **51**, 449–82. 1939.

Zymonucleic acid was extracted in relatively large quantities from living yeast cells, without affecting the nuclei at all, by washing them 2 hr. with pure water or 0.1–0.2% $NaHCO_3$ solution. With this washing, however, metachromatin granules (volutin) disappeared from the cytoplasm of *Saccharomyces ellipsoideus* and were much reduced in other yeasts. When the cells were killed with acetone, all the zymonucleic acid was extracted from the cells with the water or acetone, but still the nuclei were intact and gave positive Feulgen reactions. Thymonucleic acid extraction technics removed the material giving the Feulgen reaction from the nuclei and concentrated it in the solutions, analysis of which showed that the nuclear nucleic acid of yeasts was either thymonucleic acid or an unidentified acid very closely related to it, derived from a glucoside containing thymine, and reduced in the 2-position. The nucleus was left intact even after the second extraction and the nucleolus still stained with ferric hematoxylin.

A solution of methylene blue or cresyl blue (concentration not given), added for a few seconds either to living yeast or to smears that had been fixed in alcohol for less than ½ hr. or by flaming, stained the small metachromatin granules in the vacuoles red or reddish violet in contrast to a pale blue cytoplasm. To complete the test Meyer's reaction was used by treating the slide stained with methylene blue with 1% H_2SO_4 for a few seconds; this removes the color except from the metachromatin granules which appear black.—*V. W. Kavanagh.*

GELEI, GÁBER VON. **Neue Silbermethoden im Dienste der Protistenforschung. (Komplexsilberverbindungen).** *Zts. wiss. Mikr.*, **56**, 148–80. 1939.

The author has modified the methods of Achucarro by introducing other fixatives, reducing agents, and complex organic silver combinations. He has also substituted a sublimate–potassium-dichromate–alum mixture ($HgCl_2$, 7%; $K_2Cr_2O_7$, 2.5%; $AlK(SO_4)_2 \cdot 12H_2O$, 1%) for the usual formalin-sublimate mixture and developed a special tannin-silver technic which gives certain results in the impregnation of cilia and the basal apparatus.

A) Tannin-silver method: (Modification consists in using tannin as a mordant.) (1) Sensitize the living material in $FeCl_2$ solution prepared by adding 1 drop of 0.1% $FeCl_2$ solution to each ml. of culture fluid. The addition of 2 drops of 1% OsO_3 to every ml. of fixed solution improves the stain. (2) Fix in sublimate-dichromate-alum, 1 min. (3) Wash in dist. water, 2 changes. (4) Cold sat. soln. tannin (Merck) heated to 50° C., 2 min. (5) Wash in dist. water. (6) Place in 1% ammoniacal $AgNO_3$, ½ min. (7) Rinse in several changes of dist. water. (8) Dehydrate in alcohol series. (9) Pass thru xylene to balsam. The method gives good results with various elements of the ciliates but not with the conduction apparatus.

B) By replacing tannin in the above technic with pyrogallol-carbonic-acid (2–3–4 trihydroxy benzoic acid), three new morphological elements were stained; namely, the elastic infra-ciliary reticulum, longitudinal fibrils of the excretory apparatus, and the sheath of the neuroneme.

C) Further modifications of the ammoniacal $AgNO_3$ basic method: (1) Essential requirement: a very active *Paramecium* culture at least one week old prepared either with a boiled straw-hay infusion or fresh horse manure (1 piece to 3 liters tap water). (2) Fix in sublimate-dichromate-alum solution, 1 min. (3) Wash in 2 changes of dist. water. (4) Conc. aq. soln. pyrogallol-carbonic-acid, 2–15 min. (cold sat. soln. plus a very slight excess crystals). (5) Wash in dist. water. (6) Ammoniacal $AgNO_3$ (Rio-Hortega), 1 min. (7) Wash in 2 changes dist. water. (8) Pass thru 40% alcohol, and glycerin-alcohol; mount in glycerin alcohol. The excretory organs were demonstrated with specially good results by the introduction of other organic silver preparations such as ammoniacal silver acetate, citrate, oxalate, lactate, benzoate, salicylate, and carbonate in place of Bielschowsky's ammoniacal $AgNO_3$.—*J. M. Thuringer.*

NIETHAMMER, ANNELIESE. **Mikroskopische Bodenpilze als Begleiter in Früchten und Samen.** *Arch. Mikrob.*, **10**, 13–25. 1939.

In the course of an investigation as to the presence of fungi in seeds and small fruit, the author has developed the following technic:

Wash the fruit or seed thoroly with sterile dist. water. Section quickly, transfer the tissue pieces to a sterile slide and cover quickly with a flamed cover glass. Introduce sterile solution of Oxaminblau (further identification and strength not given) at the edge of the cover glass and allow to stain. Transfer tissue to a suitable sterile nutrient medium and allow to remain several hours. The stain carried over is not detrimental and a distinct growth of the mycelium takes place. This stain gives a good differentiation of tissue.—*Merritt N. Pope.*

STONE, W. S. and REYNOLDS, F. H. K. **A practical method of obtaining bacteria-free cultures of *Trichomonas hominis*.** *Science*, **90**, 91–2. 1939.

The isolation of the protozoon *Trichomonas hominis* is carried out in a 16 in. capillary tube drawn from an 8 in. length of 6 mm. pyrex tubing, plugged with cotton and sterilized by dry heat. The capillary is bent into a series of small traps, in the manner of a gas analysis tube. The method follows: by means of suction, fill the tube with a sterile culture liquid (Ringer's, 1 vol.; horse serum, 8 vol.); flame and seal the distal end of the capillary, and after a 48 hr. sterility

test, inoculate the medium. Incubate at 37° C., keeping the tube vertical. The protozoa will slowly migrate toward the bottom of the tube where they can be observed under the microscope, free from bacteria. Cut off and seal the capillary in a flame, drop for 1 hr. in tincture of iodine, drain off excess, and cut capillary segments into selected culture media.—*J. A. de Tomasi.*

WALTON, SETH T. **A quick and reliable method for staining Gonococcus smears.** *J. Lab. & Clin. Med.*, **24**, 1308–9. 1939.

The author gives a modification of the Pappenheim-Saathof stain. The modified solution is prepared as follows: Methyl green (dye content 60%), 1.0 g.; pyronine B certified, 0.2 g.; abs. methyl alcohol, 10 cc.; 2% aq. phenol, 100 cc.; glycerine C.P., 20 cc. Dissolve dyes by intermittent shaking for 2 days, employing a mechanical shaker. Fix films in the usual way, add dye to warm slides and let stand for 20–50 sec. The dye is selective for the *Neisseria* group.—*Sara A. Scudder.*

HISTOCHEMISTRY

ARENS, K. **Lokaler Nachweiss von Kalzium in den Membranen des Elodeablattes mittels Natriumoleat.** *Protoplasma*, **31**, (4), 508–17. 1938.

Elodea leaves remain alive for 24 hours in 0.5% solution of sodium oleate. The precipitate which gradually forms on the surfaces of the cell walls is claimed to be calcium oleate since the chemical reaction of the precipitate corresponds to that of calcium oleate.—*Robert Chambers.*

MALLORY, FRANK B. and PARKER, FREDERICK, JR. **Fixing and staining methods for lead and copper in tissues.** *Amer. J. Path.*, **15**, 517–22. 1939.

To demonstrate the presence of lead, tissues must be fixed in 95% or abs. alcohol. Formalin is worthless. The procedure recommended is: Stain celloidin sections at 54° C. for 2–3 hr., rarely longer, in the following solution: 5 to 10 mg. (not more) of hematoxylin dissolved in a few drops of abs. or 95% alcohol and mixed with 10 cc. of a freshly filtered 2% aq. solution of K_2HPO_4. After staining, wash in several changes of tap water for 10 min. to 1 hr.; dehydrate in 95% alcohol; clear in terpineol; and mount in terpineol balsam. Lead is stained a light to dark grayish blue, and nuclei a deep blue. Another method consists of staining 10–20 min. in a 0.1% solution of methylene blue in 20% alcohol. (Sources of dyes are not stated.) Differentiate 10–20 min. in 95% alcohol. The methylene blue stain works on Zenker-fixed paraffin sections and is particularly effective when phloxine is followed by methylene blue. Copper can be demonstrated after either formalin (neutral aq.) or alcohol fixation. The same hematoxylin stain is used as for lead. Hemosiderin (Fe-pigment) stains black while copper hemofuscin stains blue. The methylene blue stain colors the Cu-pigment pale blue but does not color the Fe-pigment. The probable specificity of the staining reactions was checked on tissues of animals acutely poisoned by Pb and Cu salts.—*H. A. Davenport.*

MILOVIDOV, P. **Bibliographie der Nucleal- und Plasmalreaktion.** *Protoplasma*, **31** (2), 246–66. 1938.

Almost twenty pages of references are listed on the subject of Feulgen's specific staining reaction of chromatin. Among these are included those dealing with the aldehyde reaction which may also occur in the cytoplasm.—*Robert Chambers.*

TONUTTI, E. **Ergebnisse histochemischer Vitamin C-Untersuchungen.** *Protoplasma*, **31** (1), 151–8. 1938.

The author reviews the technic of Giroud and Leblond (*Arch. Sciences et Ind.*, **435**, 1936, Paris) for cytological demonstration of vitamin C as follows: Short washing of specimen in isotonic levulose solution (5.4%); transfer to 10% $AgNO_3$ containing two drops glacial acetic per cc. (up to ½ hr.); rinse in dist. water for ¼–½ hr., changing the water; transfer to 3% $Na_2S_2O_3$, ¼–½ hr.; rinse in dist. water, ¼–½ hr.; transfer to 70% alcohol for paraffin imbedding. These steps should be performed in a dark-room with red light. Counterstain with "Kernechtrot" and light green. Sections should be thoroly dehydrated and protected from light.—*Robert Chambers.*